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Differential interactions of *eyeless* and *twin of eyeless* with the *sine oculis* enhancer

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SUMMARY

***Drosophila* eye development is under the control of early eye specifying genes including *eyeless (ey), twin of eyeless (toy), eyes absent (eya), dachshund (dac)* and *sine oculis (so)*. They are all conserved between vertebrates and insects and they interact in a combinatorial and hierarchical network to regulate each other expression. *so* has been shown to be directly regulated by *ey* through an eye-specific enhancer (so10). We further studied the regulation of this element and found that both *Drosophila* Pax6 proteins namely EY and TOY bind and positively regulate so10 expression through different binding sites. By targeted mutagenesis experiments, we disrupted these EY and TOY binding sites and studied their functional involvement in the so10 enhancer expression in the eye progenitor cells. We show a differential requirement for the EY and TOY binding sites in activating so10 during the different stages of eye development. Additionally, in a rescue experiment performed in the *so*^{1} mutant, we show that the EY and TOY binding sites are required for compound eye and ocellus development respectively. Altogether, these results suggest a differential requirement for EY and TOY to specify the development of the two types of adult visual systems, namely the compound eye and the ocellus.**

Key words: *so* enhancer, *ey*, *toy*, *Drosophila*, Eye development

INTRODUCTION

The *Drosophila* visual system consists of the two compound eyes and the ocelli, which are located on the adult vertex and consist of three simple eyes (Stark et al., 1989). Both types of eyes develop from a small number of cells that are set aside in the embryo. These cells form the eye part of the eye-antenna imaginal disc and proliferate during the larval stages. The compound eye develops from the central part whereas the ocellus develop from the anterior-medial region of the eye imaginal disc. The compound eye in *Drosophila* consists of a precisely organized array of approximately 750 ommatidia, each containing eight photoreceptor neurons and twelve accessory cells. The ommatidia form in the early third instar larva, when a wave of pattern formation, marked by an indentation called the morphogenetic furrow, moves across the eye disc in a posterior to anterior direction (reviewed by Wolff and Ready, 1993). Although committed to retinal fate, cells anterior to the furrow are still undifferentiated, whereas cells posterior to it are sequentially recruited into ommatidial clusters undergoing retinal differentiation (reviewed by Treisman and Heberlein, 1998). Our understanding of the molecular events that occur in and posterior to the furrow, such as pattern formation, ommatidial assembly and cell differentiation, has advanced dramatically in recent years. Early studies focused largely on late events of pattern formation. More recently genes involved in eye specification functioning early in eye morphogenesis have also been discovered.

Determination of the eye primordium requires several nuclear proteins that are known to act as transcriptional regulators. The *Drosophila Pax6* gene *ey* was the first gene shown to display the capacity to induce ectopic eye morphogenesis (Halder et al., 1995). Like *ey*, *toy* encodes a *Pax6* gene containing two DNA-binding domains (Czerny et al., 1999). *eye gone* (*eyg*) encodes a Pax-like protein (Jun et al., 1998), *sine oculis* (*so)* is a homeobox gene (Cheyette et al., 1994) while *eyes absent* (*eya)* and *dachshund* (*dac)* both encode different nuclear proteins (Bonini et al., 1993; Mardon et al., 1994). Analysis of the expression patterns of these genes combined with genetic approaches, have revealed a sequential and hierarchical cascade during compound eye development. *toy* is the first to be expressed during embryogenesis and activates *ey* in the eye primordium (Czerny et al., 1999). *so* is required for the development of the entire visual system, including the compound eyes, the ocelli, the optic lobe of the brain and the larval photoreceptor designated as Bolwig's organ (Cheyette et al., 1994; Pignoni et al., 1997; Serikaku and O'Tousa, 1994). *eya* is expressed later in the compound eyes and the ocelli specifying region in third instar eye imaginal discs. Like *so*, it is also required for compound eyes and ocelli formation since *eya* mutants lack both visual systems

(Zimmerman et al., 2000). *so*, *eya* and *dac* have been shown to be downstream of *ey* and regulated by it (Halder et al., 1998; Niimi et al., 1999; Zimmerman et al., 2000). These proteins form complexes that feed back on *ey* expression and they are also capable of inducing ectopic eye morphogenesis (Bonini et al., 1993; Bonini et al., 1997; Pignoni et al., 1997). Despite a high sequence homology in their DNA binding domains, EY and TOY appear to exhibit different biological roles during development (Czerny et al., 1999). First, these genes are expressed differentially during embryonic development. Second, *ey* and *toy* are co-expressed in the eye imaginal disc but only *toy* is expressed in the ocellar region. Therefore, the lack of *ey* in ey^2 mutants impairs compound eye formation but not ocellar development. This suggests that *so* and *eya* are not under the control of *ey* in the ocelli but require other regulators to ensure their proper expression in these cells. The precise role of *toy* during *Drosophila* development is still not understood.

Analysis of elements that control the expression pattern of genes involved in early eye development should provide additional details on the genetic hierarchy during eye specification. It has been shown that *toy* induces the expression of *ey* through the eye-specific enhancer of the *ey* gene in the eye precursor cells of the embryo, but not during the larval stages in the eye disc (Czerny et al., 1999). The *so*10 enhancer, which is part of an eye-specific regulatory sequence deleted in the so^1 mutant, has been shown to be a direct *ey* response element (Niimi et al., 1999). Recently, genomic deletions proximal to the promoter found in eya^1 and eya^2 mutants were identified as eye-specific enhancers. These elements have been shown to be inducible by EY in the antennal disc (Zimmerman et al., 2000). All these data provide additional information leading to a better understanding of the complexity of the interacting network during early eye development.

In this study, we addressed the regulation of the *so*10 enhancer during eye morphogenesis. We show that the *so*10 enhancer is bound and regulated by EY and TOY through their paired domain (PD). Despite extensive sequence homology, the PD of EY and TOY bind different sites in this enhancer. Targeted mutagenesis experiments allowed us to establish that these different binding sites are functional and required to ensure proper activity of the *so*10 enhancer in the eye disc. Finally, rescue experiments of the so^1 mutant using the different mutated versions of the *so*10 enhancer demonstrated that TOY and EY have different functions in the formation of the compound eyes and the ocelli through the same enhancer. Thus the TOY binding sites are absolutely required for ocelli development and the EY binding sites are required for compound eye formation.

MATERIALS AND METHODS

Fly strains and histology

Flies were reared on standard medium at 25°C. Lines used: *so*10-*lacZ* (Niimi et al., 1999), dpp^{blink}-Gal4 (Staehling-Hampton and Hoffmann, 1994), UAS-*ey* (Halder et al., 1995), UAS-*toy* (Czerny et al., 1999), ey^2 (Quiring et al., 1994), so^1 (Cheyette et al., 1994), eya^1 (Bonini et al., 1993), spa^{pol} (Fu and Noll, 1997), UAS-*eyΔPD* and UAS-*eyΔHD* (Punzo et al., 2001), $ey^{J5.71}$ and $toy^{G7.39}$ (S, F., U. Kloter, and W. J. G., unpublished).

Specific genotypes were generated: (1) *so*10-lacZ/*so*10-lacZ; dpp^{blink}-*Gal4/TM6B,Tb,Hu*; spa^{pol}/spa^{pol}, (2) *so*10-*lacZ*/*so*10-lacZ; ey^2/ey^2, (3)so^1/so^1; *so*10-*lacZ*/*so*10-*lacZ*, (4) eya^1/eya^1; *so*10-*lacZ*/*so*10-*lacZ*, UAS-*toy*/UAS-*toy*; $ey^{J5.71}/ci^D$, UAS-*toy*/UAS-*toy*; ey^2/ey^2. Transgenic lines were generated by P-element-mediated germline transformation in yw^{1118}. New lines were created for this study: $so10^{EY/TOYmt}$, $so10^{TOYmt}$, $so10^{EY+TOYmt}$-Gal4 and $so10^{EY+TOYmt}$-*lacZ* lines; *so*9-*lacZ*, *so*7-*lacZ*, *so*7-Gal4, $so7^{EY/TOYmt}$-Gal4, $so7^{TOYmt}$-Gal4, $so7^{EY+TOYmt}$-Gal4; UAS-*toy*ΔPD and UAS-*toy*ΔHD. For each construct at least two lines with P-element insertions onto different chromosomes were analysed to ensure correct expression patterns. Each Gal4 line was verified for correct expression patterns by crossing to a UAS-*lacZ* followed by X-gal staining on discs. *lacZ* expression was detected by X-gal staining on discs, or by means of immunohistochemestry with a monoclonal anti-β-gal antibody (Promega) according to Halder et al. (Halder et al., 1998).

Gel shift and footprinting assays

Gel shift assays were performed with the 128 bp *so* fragment and full-length EY and TOY proteins as described previously (Niimi et al., 1999). Full-length EY and TOY proteins were synthesized in reticulocyte lysates from the T7 and T3 promoter, respectively, according to the manufacturer specification (Promega) by using the pBSK-*ey* and *toy* plasmids (Czerny et al., 1999; Quiring et al., 1994). For the DNaseI footprinting assay, 6xHis tagged paired domains (pQE30-EY PD and TOY PD) were produced and purified in native condition onto Ni-NTA columns according to the manufacturer's specifications (Qiagen). The proteins were diluted in the binding buffer to obtain a final concentration of 10 mM Tris pH7.5, 75 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10% glycerol, 25 mM imidazole, and 50 μg/ml poly(dI-dC). Reactions were carried out in 100 μl by incubating 100 ng of His-PD purified proteins with 10 ng of the *so*10 fragment end-labelled with [α-^{32}P]dATP at *Asp*718 for 15 minutes at room temperature followed by digestion with 0.001 U of DNaseI (Boehringer Manheim) for 1 minutes at 20°C. After phenol-chloroform extraction, analysis was performed on a denaturing sequencing gel followed by autoradiography.

Western blot experiments

Western blot experiments were done with a rabbit anti-EY antibody or with a rabbit anti-TOY antibody at a dilution of 1:200. The antibody was preabsorbed with larval tissue. Each lane was loaded with extracts from 10 leg discs. All extracts for western blotting were boiled for 6 minutes. Transfer was verified by Ponceau Red staining. The secondary antibody for detection of the signal was used at a dilution of 1:2000 (HRP-coupled swine anti-rabbit antibody from DAKO A/S) and the signal was revealed using a chemoluminescence kit according to the manufacture's specifications (Amersham).

Cloning procedure and plasmids

The EY and TOY binding sites within the *so*10 enhancer were sequentially mutated using standard PCR amplification procedures. The resulting 428 bp *Asp*718-*Eco*RI mutated fragments were subcloned in pBluescript SK, sequenced, further excised using *Bam*HI-*Asp*718 and subcloned into the *lacZ* pCβ vector (Niimi et al., 1999) at *Asp*718-*Bam*HI. To generate *so*10 and *so*7-GAL4 expression vectors, we first modified the pUAST vector (Brand and Perrimon, 1993) by deleting the *Xba*I-*Sph*I fragment, removing the polylinker, UAS sequences and the hsp70 promoter. A new polylinker was inserted through the same sites to create the new *Asc*I-*Not*I-*Spe*I-*Bgl*II-*Kpn*I-*Ngo*MIV-*Eag*I-*Eco*RI-*Avr*II-*Nhe*I-*Sph*I multiple cloning sites. The Gal4 encoding sequence was amplified by PCR in order to create *Asc*I-*Not*I cloning sites at both ends and subcloned into the modified pUAST vector to generate the pP-Gal4 vector. A 300 bp *Not*I minimal hsp70 promoter fragment was further inserted in front of the Gal4 at *Not*I. This resulted in the pPhsp70-Gal4 vector. The 428 bp *so*10 enhancer and derivatives were inserted into this pPhsp70-Gal4 vector at *Eco*RI-*Asp*718 to generate the resulting *so*10-Gal4

constructs. To generate the *so*7 constructs, the 1.6 kb *Eco*RI *so*7 fragment (Niimi et al., 1999) was first subcloned into Bluescript SK at *Eco*RI. Then, the *Asp*718 1.2 kb subfragment *so*9 was excised and subcloned at *Asp*718 in the correct orientation into the different derivatives of the *so*10-hsp70-Gal4 constructs resulting in the *so*7-Gal4 constructs. The *so*9-*lacZ* construct was generated by cloning the 1.2 kb *Asp*718 *so*9 fragment into pCβ (Niimi et al., 1999) at *Asp*718. The 128 amino acids EY- and TOY-PD were amplified by PCR in order to create *Bgl*II and *Asp*718 cloning sites at both ends to ensure cloning in the correct ORF into the pQE30 vector at *Bam*HI-*Asp*718 sites (Qiagen). After PCR amplification, each construct was verified by sequencing. The *toy* cDNA was deleted between P_{13}-Q_{162} for *toyΔPD* using the *Nsi*I-*Blp*I sites to generate the *toyΔPD*. The *toyΔHD* was deleted between L_{219}-R_{291} using *Bbs*I-*Eag*I sites. The deleted cDNAs were further excised from Bluescript and inserted as an *Asp*718-*Xba*I fragment in pUAST. Detailed description of the primers used will be given upon request.

RESULTS

EY and TOY are involved in *so*10 enhancer expression

Recent genetic analysis demonstrated that the induction of *so* mediated by EY occurs during second instar larvae in the eye progenitor cells (Halder et al., 1998). A genomic fragment deleted in the so^1 mutant has been shown to interact specifically with *ey* in a yeast one hybrid assay and to be ectopically inducible by EY in vivo (Niimi et al., 1999). Therefore, we were interested in the regulation of this enhancer element (*so*10) during normal eye development. To address this question, we first studied the expression of the *so*10 enhancer in an *ey* mutant background. When compared to a wild-type eye disc (Fig. 1A), the expression of the transgene (*so*10-*lacZ*) is dramatically reduced in the anterior to posterior central region of an ey^2 mutant eye disc (Fig. 1B) and also of an *ey* null mutant eye disc (Fig. 1C) (*ey* null: $ey^{J5.71}$; S. F., U. Kloter and W. J. G., unpublished) (Punzo et al., 2001). The residual lateral expression of the enhancer detected in the eye disc indicates that additional transcription factors are involved in *so*10 regulation. To investigate whether the loss of expression is due to a loss of EY rather than to a loss of *so*10-expressing cells by apoptosis or a loss of the expression of *ey* downstream genes, we studied the expression of the *so*10 enhancer in so^1 and eya^1 mutants. Like *ey* mutants these two mutants also exhibit an increased amount of apoptosis (reviewed by Treisman and Heberlein, 1998). As shown in Fig. 1D,E the expression in the anterior region of the eye disc is not affected in so^1 and eya^1 mutants, respectively. These results strongly support the idea that the loss of expression detected in the anterior part of an ey^2 or an $ey^{J5.71}$ mutant eye disc is directly due to a loss of EY and not to the loss of expression of *ey* downstream genes. The loss of expression in the posterior region shows that this enhancer is regulated differently in the anterior than in the posterior part of the eye disc. It suggests a concerted involvement of EY/SO/EYA in the expression of this element in differentiating cells, which has not been further investigated in this study.

The *sparkling* (*spa*) gene, which is involved in lens formation, belongs to the Pax2/5/8 gene family and is expressed posterior to the morphogenetic furrow (Fu and Noll, 1997). We also investigated a possible involvement of this Pax gene in *so*10 enhancer regulation posterior to the morphogenetic furrow. As shown in Fig. 1F the expression of *so*10 seems not to be affected in this mutant. *toy*, the second *Pax6* gene expressed in the developing eye disc, reveals a similar expression pattern to *ey* (Czerny et al., 1999). Since the *so*10 enhancer exhibits residual expression in *ey* mutants, we asked whether TOY could also be involved in its regulation. We addressed this question by using the recently isolated *toy* mutant $toy^{G7.39}$ (S. F., U. Kloter and W. J. G., unpublished). Owing to the hypomorphic character of this mutant the size of the eye disc can vary a lot within the same larva from a small cluster of cells to almost wild-type size (Fig. 1I). According to

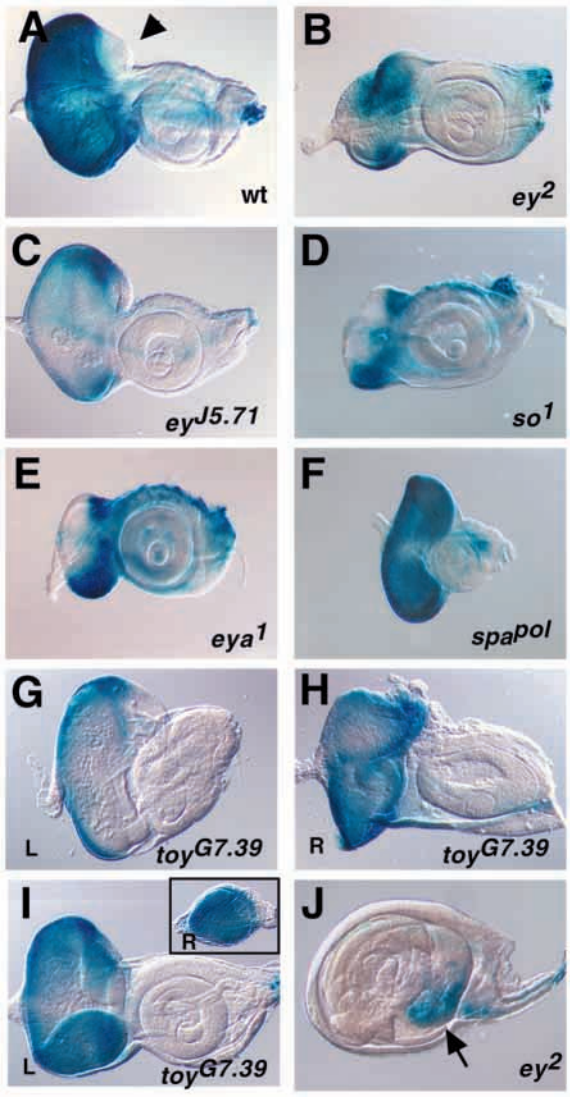

Fig. 1. *so*10-*lacZ* activity in different mutant backgrounds. (A-I) Eye discs of third instar larvae expressing *so*10-*lacZ*. (A) Expression in a wild-type eye disc. Arrowhead indicates the ocellus region where no expression is detected. Dorsal is top and posterior is left. (B-J) Expression in an ey^2, $ey^{J5.71}$, so^1, eya^1, spa^{pol} and $toy^{G7.39}$ mutant background, as indicated. G and H, I and upper box in I show a pair of the eye discs from a single larva of $toy^{G7.39}$. L indicates left and R indicates right eye disc. (J) Leg disc where UAS-*toy* is ectopically expressed with dpp^{blink}-Gal4 in an ey^2 mutant background. *lacZ* staining (arrow) shows that *toy* is able to activate this enhancer element in the absence of endogenous *ey*.

this, *toy* transcript is still detected in eye discs of normal size whereas it is not detected in strongly reduced eye discs (data not shown). As shown in Fig. 1G-I *so*10-*lacZ* expression is reduced in the central region of a $toy^{G7.39}$ mutant eye disc. Whereas in Fig. 1G the expression is restricted to the posterior margin of the eye disc, in Fig. 1H a clear territory is not definable owing to the shape of the eye disc although both discs derive from the same larva. These results suggested to us that *toy* is involved in *so*10 regulation but because of a high variability of the *toy* transcript (data not shown), and therefore a high variability in eye disc size and shape (Fig. 1G-1I), it was not possible to asses a clear *toy*-dependent territory. To further corroborate an involvement of TOY in *so*10 regulation we switched to ectopic expression experiments using the UAS-GAL4 system (Brand and Perrimon, 1993). We performed experiments in an ey^2 mutant where ectopic expression of *toy* is not able to induce *ey* (Czerny et al., 1999). As shown in Fig. 1J, ectopic expression of TOY in ey^2 induces expression of the *so*10 enhancer. This led us to the conclusion that *so*10 enhancer might be regulated by both, EY and TOY in *Drosophila*, and that the residual staining found in ey^2 or $ey^{J5.71}$ eye discs could be in part due to the activation by *toy*.

EY and TOY activate the *so*10 fragment directly

To determine whether TOY directly binds to the *so*10 fragment in vitro, we performed bandshift experiments using full-length EY and TOY on a 128 bp *so*10 sub-fragment previously shown to be bound by EY (Niimi et al., 1999). As shown in Fig. 2A TOY is able to bind specifically to this fragment since a 10-fold molar excess of cold competitor inhibits binding. This result further enforced our finding that *toy* is also able to regulate *so*10 and encouraged us to study this regulation.

Pax6 proteins contain two DNA binding domains, the paired domain (PD) and the homeodomain (HD). It has recently been shown that the EY-PD is required to induce endogenous *so* expression and to direct eye development, whereas the EY-HD is dispensable for this process (Punzo et al., 2001). Therefore, we investigated whether the same mechanism applies to *so*10 regulation. We used EY- and TOY-deleted proteins in which the PD or the HD was missing. To ensure that the TOY constructs would not activate endogenous *ey* that in turn would activate *so*10, we performed the experiment in an ey^2 mutant background, as previously described for full-length *toy*. Ectopic expression of these proteins in the appendages by dpp^{blink}-Gal4 revealed that the HD deletion has no effect on *so*10 inducibility (Fig. 2E,H) when compared to the full-length proteins (Fig. 2D,G), whereas the removal of the PD domain abolished the ability of both EY and TOY to induce *so*10 expression (Fig. 2F,I). To ensure that the deleted proteins were expressed at comparable levels, we performed western blotting experiments probed with an anti-TOY or an anti-EY antibody, respectively. As shown in Fig. 2B,C the deleted proteins (asterisk) are expressed at comparable or lower levels than the full-length TOY or EY proteins. Despite a lower level for the HD deleted constructs these proteins still induce *so*10-*lacZ* whereas the PD-deleted protein does not. Altogether, these results demonstrate that EY and TOY activate the *so*10-*lacZ* transgene through their paired domain.

These findings prompted us to identify the PD binding sites of EY and TOY within this element. We performed in vitro DNase I footprinting experiments using His-tagged EY and TOY PD. As shown in Fig. 3B, three protected regions were found by using the EY-PD whereas five protected regions were found by using the TOY-PD. Whereas sites 3 and 4 are only protected by TOY, sites 1,2 and 5 are equally bound by EY and TOY in vitro. Therefore, we were not able to find specific binding sites protected exclusively by EY, in contrast to TOY. As shown in Fig. 3C, these protected sites reveal sequence similarity to the in vitro selected Pax6 consensus binding sequence (Czerny and Busslinger, 1995; Epstein et al., 1994). To address the question of the functional relevance of these binding sites, we mutated the different binding sites and studied the effect of the mutations on the enhancer activity. We mutated the bases fitting with the Pax6 consensus in order to lose the core homology (Fig. 3A). Since the *Pax6* consensus is bipartite and highly degenerated it was necessary to introduce several mutations within each binding site to ensure the complete loss of EY-PD and TOY-PD binding, respectively

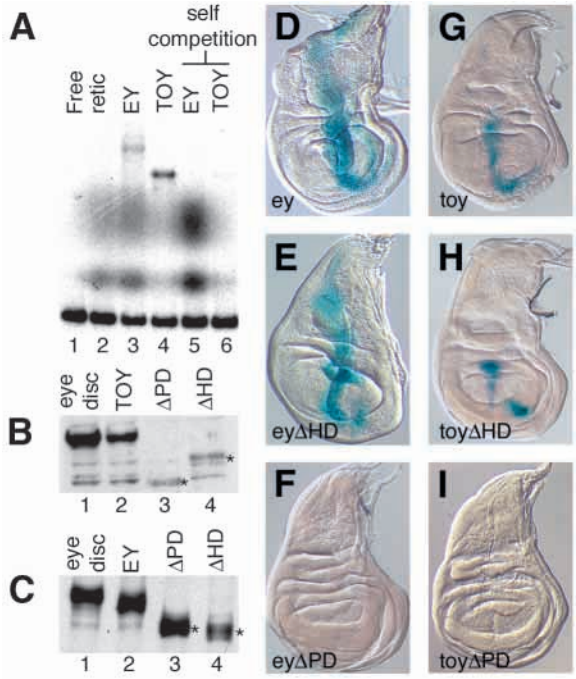

Fig. 2. EY and TOY bind to the *so*10 enhancer through their paired domains. (A) Bandshift experiment performed with equal amounts of in vitro synthesised full-length EY and TOY on a 128 bp fragment from the 428 bp enhancer of *so*10. EY and TOY are both able to bind specifically to this fragment since binding is abolished with a 10-fold molar excess of cold competitor (compare lanes 3, 4 with lanes 5, 6). (B,C) Western blot analysis of the ectopically expressed EY and TOY proteins in third instar leg discs with an anti-TOY (B) and an anti-EY (C) antibody. Lanes 1: *yw* control eye discs; lanes 2: misexpression of full-length *toy* and *ey*; lanes 3: misexpression of *toyΔPD* and *eyΔPD*; lanes 4: misexpression of *toyΔHD* and *eyΔHD*. Asterisk indicates the deleted proteins. (D-I) Third instar wing discs in which different UAS-*ey* or UAS-*toy* constructs are misexpressed with dpp^{blink}-Gal4 in an ey^2 mutant background. The X-gal staining reveals the ability of these proteins to induce *so*10. (D,G) Full-length EY and TOY, respectively, are able to induce *so*10. (E,H) Deletion of the HD in EY or TOY, respectively, does not abolish *so*10 activation. (F,I) Deletion of the PD of EY or TOY, respectively, completely abolishes *so*10 activation. Note that despite a lower level of ΔHD proteins *so*10-*lacZ* is still significantly activated.

(Fig. 3A: mutation of binding sites 1 to 5 referred to as *so*10$^{EY+TOYmt}$). In addition, after generating transgenic lines carrying the enhancer with all five binding sites mutated, we verified that this element was not inducible anymore by EY and TOY in wing (Fig. 3D) and leg discs (data not shown). These results strongly suggest a direct role of these proteins in *so*10 activation through the five identified binding sites.

EY and TOY proteins play a different roles in *so*10 enhancer regulation during eye development

To correlate the different binding properties of EY and TOY on the *so*10 enhancer to their function during eye development, we generated two additional *so*10-*lacZ* transgenic lines. One line carries mutations in TOY binding sites 3 and 4 referred to as *so*10^{TOYmt}. The other carries mutations in the EY and TOY binding sites 1, 2 and 5 referred to as *so*10$^{EY/TOYmt}$.

The expression of these different *so*10-*lacZ* constructs was further analyzed with regard to eye development during the larval stages. As shown in Fig. 4C and 4G, when the two TOY-specific binding sites 3 and 4 are disrupted (*so*10$^{TOY\ mt}$), the expression is maintained in the entire eye disc in early third instar larvae, and only later becomes restricted to the posterior side. The expression is completely lost in *ey*^{2} mutants (data not shown) suggesting that the residual staining is due to EY activity. Disrupting the EY/TOY binding sites (*so*10$^{EY/TOYmt}$) consequently targets the expression of the transgene in the lateral edge of the eye disc (Fig. 4D,H). Interestingly, the expression patterns of *so*10$^{TOY\ mt}$ and *so*10$^{EY/TOYmt}$ mimic the expression patterns obtained with the wild-type *so*10 enhancer in a *toy*$^{G7.39}$ and in an *ey*^{2} or *ey*$^{J5.71}$ mutant background (Fig. 1G, 1B and 1C, respectively). Thus during larval stages, completely removing EY gives the same result as disrupting

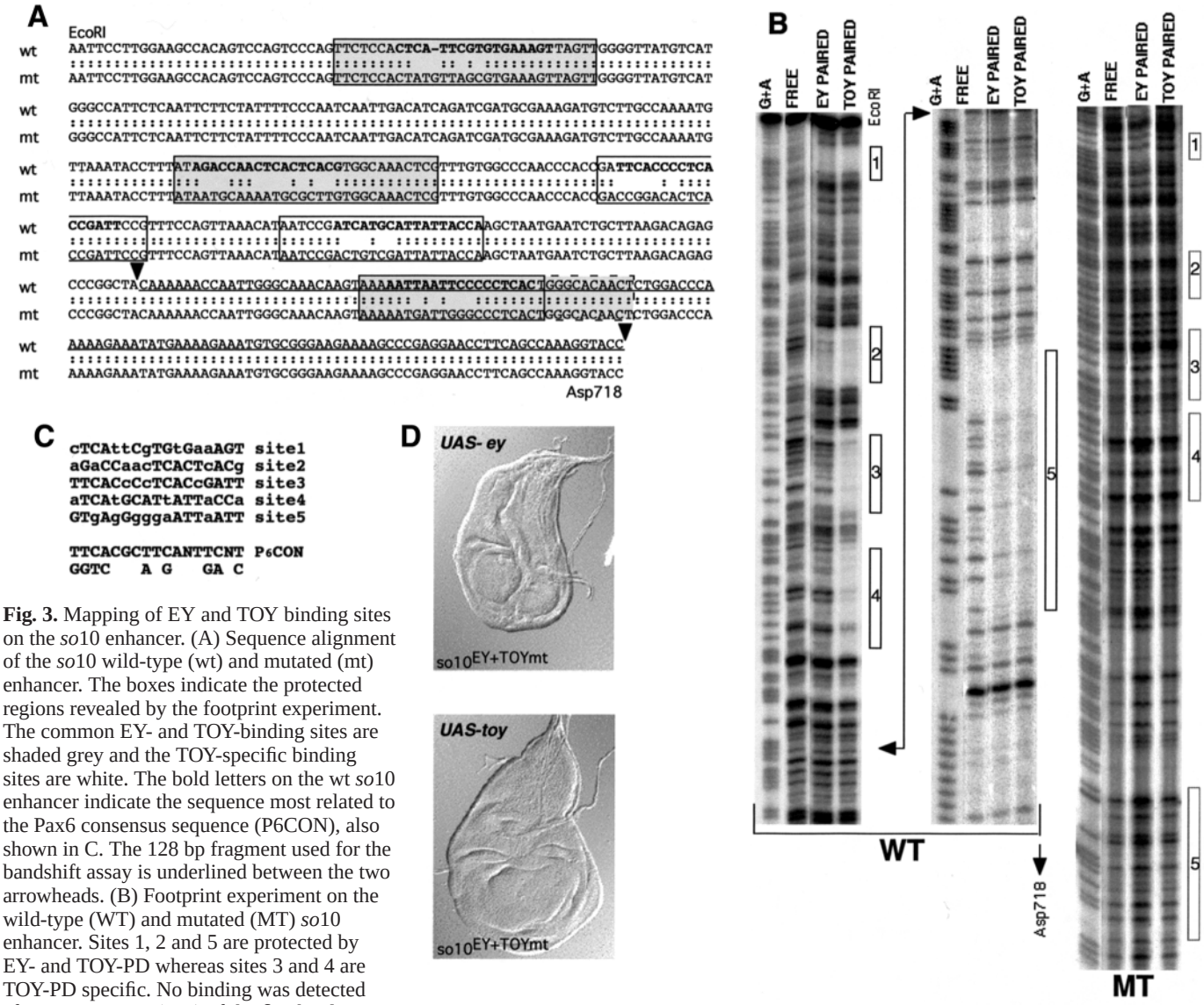

Fig. 3. Mapping of EY and TOY binding sites on the *so*10 enhancer. (A) Sequence alignment of the *so*10 wild-type (wt) and mutated (mt) enhancer. The boxes indicate the protected regions revealed by the footprint experiment. The common EY- and TOY-binding sites are shaded grey and the TOY-specific binding sites are white. The bold letters on the wt *so*10 enhancer indicate the sequence most related to the Pax6 consensus sequence (P6CON), also shown in C. The 128 bp fragment used for the bandshift assay is underlined between the two arrowheads. (B) Footprint experiment on the wild-type (WT) and mutated (MT) *so*10 enhancer. Sites 1, 2 and 5 are protected by EY- and TOY-PD whereas sites 3 and 4 are TOY-PD specific. No binding was detected after mutagenesis (MT) of the five binding sites. (C) Sequence alignment of the five binding sites identified on *so*10 with the Pax6 consensus binding site (P6CON) (Epstein et al., 1994). The bases fitting with the consensus are shown in capital letters. (D) Wing discs where either EY or TOY were misexpressed by *dpp*^{blink}-Gal4 in flies carrying the *lacZ* reporter gene under the control of the *so*10$^{EY+TOYmt}$ enhancer. In both cases no β-galactosidase expression was detected indicating that the mutated enhancer was not inducible anymore by TOY or EY.

the EY/TOY binding sites whereas partially removing TOY (hypomorph) leads only in some cases (Fig. 1G) to the same result as disrupting the TOY binding sites. Mutation of the five binding sites (*so*10$^{EY+TOYmt}$) abolishes any residual expression in the eye disc (Fig. 4E,I) suggesting a role for both EY and TOY in specifying expression of *so*10 in the eye disc.

The *so*10 eye enhancer restores eye development in the *so*^{1} mutant

To define functional sites within the *so*10 enhancer, we tried to correlate the different expression patterns observed to their requirements. We therefore asked whether the different mutated enhancers had the ability to rescue the *so*^{1} mutant phenotype, which lacks both compound eyes and ocelli, and to what extent this phenotype can be rescued. We used the UAS-Gal4 system to generate transgenic flies expressing the Gal4 transcription factor under the control of the different mutated enhancers. Those lines were then crossed to a UAS-*so* line in a *so*^{1} mutant background (Fig. 5A). First we confirmed that the different *so*10-Gal4 drivers directed expression of a UAS-*lacZ* reporter transgene in similar manner to that obtained with the *so-lacZ* constructs previously examined (data not shown). Then we tested if the wild-type *so*10 element is sufficient for rescuing the *so*^{1} mutant eye phenotype. As shown in Fig. 5B, expression of the *so* cDNA under the control of the *so*10 fragment in a *so*^{1} mutant rescued the compound eyes effectively but failed to rescue the missing ocelli. This result is consistent with the expression pattern of this element showing expression in the eye-specifying region but lacking expression in the ocellus region (Fig. 1A arrowhead). Thus, the transgene contains appropriate DNA regulatory sequences in order to direct and restore *so* expression in compound eye formation in a *so*^{1} mutant.

We next asked how far the mutated Gal4 drivers were also able to restore proper *so* expression in a *so*^{1} mutant background and therefore rescue eye development. As shown in Fig. 5C, mutations introduced in the TOY binding sites (*so*10^{TOYmt}) have only a minor effect since the eye is rescued to approximately the same size as the rescue obtained with the wild-type *so*10 enhancer. However, disruption of the EY/TOY binding sites (*so*10$^{EY/TOYmt}$), which largely inactivated the enhancer (Fig. 4D and H), gave hardly any rescue (Fig. 5D). Indeed, we observed in almost 100% of the offspring a partial rescue only, characterized by a strongly reduced eye on one side and no eye on the other side of the fly head. This phenotype is rarely seen in *so*^{1} mutants. Interestingly, it is reminiscent and strongly resembles the eye phenotype observed in the *ey*^{2} mutant (Halder et al., 1998). It has recently been shown by Punzo et al. (Punzo et al., 2001) that the variable eye phenotype is not due to a residual expression of *ey*. The expression of EY is not detectable in the eye disc of *ey*^{2} mutants and the same phenotype is observed in the *ey* null mutant *ey*$^{J5.71}$. Thus, the presence of the TOY binding sites within the *so*10$^{EY/TOYmt}$ enhancer allows a partial rescue, suggesting that TOY can overcome the lack of EY function to some extent. Therefore, this finding might explain why *ey* mutant flies have a strong variation in eye size. Finally, the *so*10$^{EY+TOYmt}$ enhancer did not rescue the *so*^{1} mutant phenotype (Fig. 5E). These results suggest a direct role for the EY/TOY binding sites in *so*10 regulation during compound eye development, whereas the TOY binding sites seem to contribute only little to the formation of the compound eye.

To further investigate whether TOY can overcome the lack of EY, we tried to rescue the eye phenotype of an *ey* mutant by TOY. Flies carrying either the UAS-*ey* or the UAS-*toy* cDNA were crossed to *ey*-enhancer-Gal4 flies in an *ey*$^{J5.71}$ mutant background and the offspring was examined. As shown in Fig. 5H, *toy* was also able to rescue an *ey*$^{J5.71}$ mutant eye phenotype if expressed under the control of the *ey* enhancer. This demonstrates that in the absence of *ey*, *toy* can partially complement *ey*, and suggests that the two Pax6 homologues have partially redundant functions.

Different requirement for the TOY- and EY-binding sites during development

Since the rescue obtained with the *so*10 enhancer was incomplete and resulted in a reduced compound eye (Fig. 5B), irregular ommatidia arrangement, lack of inter-ommatidial bristles and of ocelli (data not shown), we investigated whether additional regulatory sequences on the adjacent 1.2 kb fragment, encompassing the deletion in *so*^{1}, were required for proper *so* expression. Therefore, we generated a new *so*-Gal4 driver, which contained the 1.2 kb fragment adjacent to the 400 bp *so*10 element, in the same orientation as found in the endogenous gene. This construct was designated as *so*7 driver (the 1.2 kb fragment corresponds to *so*9, whereas *so*7 includes fragments *so*9 + *so*10) (Niimi et al., 1999). The *so*9 fragment was not activated by EY in a yeast one hybrid assay, whereas *so*7 containing the *so*10 was (Niimi et al., 1999). The mutated *so*7-Gal4 drivers were generated using the same mutations as previously described for the various mutated *so*10 enhancers,

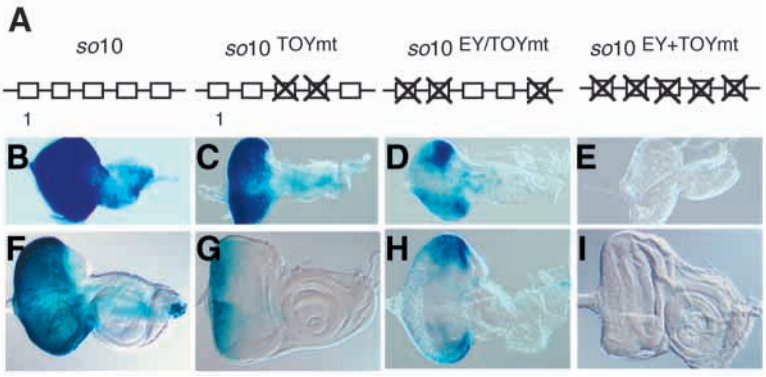

Fig. 4. Expression pattern of the different *so*10 enhancers. (A) Schematic drawing of the enhancer with the different EY and TOY binding sites indicated by five boxes. The different mutated *so*10 enhancers used are represented using crossed boxes. All panels in a column refer to the same *so*10 enhancer. (B-E) Early third instar eye discs and (F-I) late third instar eye discs. During larval stages the *lacZ* transgene was detected by staining for β-galactosidase. For all eye discs, posterior is left and dorsal is towards the top. During early third instar larval stage the expression is lost in the central part of the eye disc when the EY binding sites are mutated (D), in contrast to mutations of the TOY binding sites which show no obvious defects at this stage (C). At late third instar stage mutations of the TOY binding sites restrict expression to the posterior edge of the eye disc (G) whereas mutations of the EY binding sites do not alter the expression pattern from second to third instar larvae (D,H). Expression is completely abolished in the eye primordium and in the eye disc when all five binding sites are mutated (E,I).

and are referred to as *so*7$^{EY/TOYmt}$, *so*7^{TOYmt} and *so*7$^{EY+TOYmt}$, respectively. As a control, we generated a *lacZ* line carrying only the *so*9 fragment to test whether this element is also expressed in the eye disc. As revealed by *lacZ* staining, a weak expression was detectable at the posterior margin of the eye disc (data not shown). When crossed to UAS-*lacZ*, the *so*7 driver extended the expression further into the ocellar region of the eye disc (Fig. 6B) than the *so*10 driver (Fig. 4F). The *so*7$^{EY/TOYmt}$ line produced expression in the ocellar region but not in the eye-specifying region except at the posterior margin of the eye disc, which was caused by to the *so*9 fragment (Fig. 6C). This result indicates that the expression in the eye-specifying region depends on the presence of the EY/TOY binding sites 1, 2 and 5. Compared with the *so*10$^{EY/TOYmt}$ (Fig. 4H), the *so*7$^{EY/TOYmt}$ is not expressed in the lateral edges of the eye disc, showing that *so*9 restricts the activity of *so*10 through the TOY binding sites. The *so*7^{TOYmt} enhancer (Fig. 6D) shows a similar expression pattern as the *so*10^{TOYmt} enhancer (Fig. 4G) in the posterior part of the eye disc. In addition, the *so7*-enhancer-specific expression in the ocellar region is missing in *so*7^{TOYmt} indicating that it is dependent on the TOY binding sites. In summary, in the context of the *so*7 enhancer, disrupting the EY/TOY binding sites affects expression mainly in the eye field whereas disrupting the TOY binding sites affects the expression in the ocelli. Thus, *so*9 cooperates with the *so*10 enhancer to specify expression in the ocelli through the TOY binding sites. Finally, when all five binding sites were mutated (*so*7$^{EY+TOYmt}$) only staining reflecting the pattern of *so*9 was detected (Fig. 6E). Overall, the expression patterns of the *so*7-Gal4 lines confirmed our previous findings on the *so*10 element and further attested a different requirements for the EY and TOY binding sites.

To correlate the function of the *so*7 enhancers with different expression patterns, we performed a rescue experiment by restoring *so* expression using the UAS-Gal4 system in a *so^{1}* mutant background. We crossed the different *so*7-Gal4 enhancers to UAS-*so* in a *so^{1}* mutant background and tested their ability to rescue the *so^{1}* mutant phenotype. As shown in

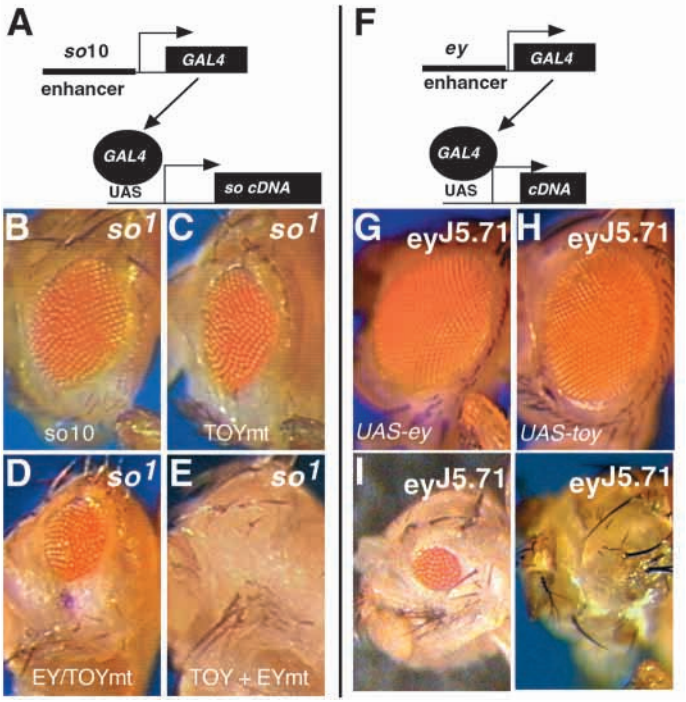

Fig. 5. Rescue of the *so^{1}* mutant by the different mutated *so*10 enhancers. (A) Schematic drawing of the Gal4 system used for the rescue experiment. Flies expressing the Gal4 gene under the control of the different *so*10 enhancers were crossed to flies carrying the UAS promoter controlling the *so* cDNA. The crosses were all carried out in a *so^{1}* mutant background. (B,C) Rescue of the *so^{1}* mutant eye phenotype with *so*10 and *so*10^{TOYmt}, respectively. (D) Rescue obtained with the *so*10$^{EY/TOYmt}$ enhancer. The eye size shown is the largest obtained. The variations in size were reminiscent of those observed in an *ey^{2}* mutant with one eye missing. (E) No rescue was observed with the *so*10$^{TOY+EYmt}$. (F) Schematic drawing of the Gal4 system used to rescue the *ey* null mutant. (G,H) Rescue of the *ey* null mutant *ey$^{J5.71}$* with the *ey*-enhancer Gal4. Both UAS-*ey* (G) and UAS-*toy* (H) were able to rescue the *ey$^{J5.71}$* mutant when driven with *ey*-Gal4. In both cases (G,H), the eyes were almost normal in size for all of the flies analysed. The *ey$^{J5.71}$* null mutant showed an eye reduction of between 50-75% for 20% of the flies (I), and 75%-100% for 80% of the flies (J) (Callaerts et al., 2001).

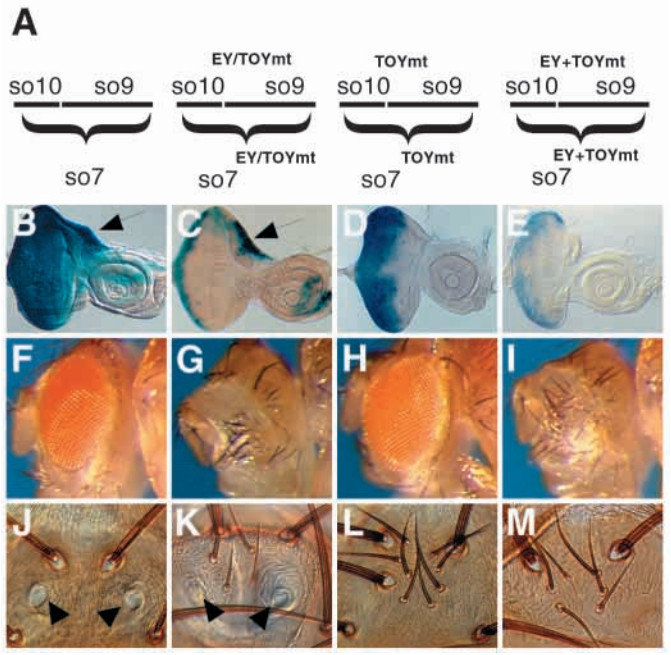

Fig. 6. Rescue of the *so^{1}* mutant phenotype by the 1.6 kb element *so7*. (A) Schematic representation of the different *so*7 drivers in combination with the previously described *so*10 mutated elements. (B-E) Expression pattern of the different *so*7 drivers that have the extended *so*9 fragment. All panels show third instar eye discs with posterior to the left and dorsal to the top. (B) *so*7 shows additional expression in the ocellus region (arrow). (C) *so*7$^{EY/TOYmt}$ completely loses expression in the eye disc but still has an extended expression in the ocellus region (arrow). (D) *so*7^{TOYmt} has normal expression in the eye disc but not in the ocellus region. (E) *so*7$^{EY+TOYmt}$ only shows expression reminiscent of the additional 1 kb element at the posterior margin of the eye disc. (F-I) Rescue of the adult eye with the *so*7 drivers. The wild-type *so*7 and the *so*7^{TOYmt} show full rescue of the compound eye (F,H), whereas the removal of the EY binding sites does not rescue, or only partially in very rare cases (G). *so*7$^{EY+TOYmt}$ never shows any kind of rescue (I). (J-M) Rescue of the ocelli with the *so*7 drivers. In contrast to the compound eyes, the TOY binding sites are absolutely required for ocelli development. (J,K) Rescue of the ocelli (arrowheads) with *so*7 (J) and *so*7$^{EY/TOYmt}$ (K). No rescue is observed with *so*7^{TOYmt} (L) and *so*7$^{EY+TOYmt}$ (M).

Fig. 6F, *so*7 was able to fully rescue the compound eyes and partially rescue the ocelli (Fig. 6J). *so*7$^{EY/TOYmt}$ was not able to restore compound eye development (Fig. 6G) but was still able to direct ocelli formation (Fig. 6K). In contrast, *so*7^{TOYmt} rescued compound eye formation (Fig. 6H) at the same efficiency as the wild-type enhancer, but did not rescue ocelli development (Fig. 6L). *so*7$^{EY+TOYmt}$ was unable to rescue either the compound eye or the ocelli phenotype (Fig. 6I and M). This demonstrates that the EY/TOY binding sites are absolutely required for compound eye development whereas the TOY binding sites are required for the development of the ocellus. Thus, these results suggest a distinct functions for the two *Drosophila* Pax6 proteins in regulating the same target gene during compound eye and ocellus development.

DISCUSSION

so, ey and *eya* regulate the *so*10 enhancer in a complex network

Genes involved in eye development are highly conserved between vertebrates and invertebrates. Given the complex genetic network during early eye development, studies of regulatory sequences controlling eye-specific expression provide insight into eye specification. Analyzing the expression of the eye-specific *so*10 enhancer in different mutant background gives information about the regulatory circuit. The loss of *so*10-*lacZ* expression in the ey^2, $ey^{J5.71}$ or $toy^{G7.39}$ mutants indicates, that EY and TOY are required for activation of this element. In the case of *so* and *eya*, the analysis in the null mutant was not possible due to embryonic lethality of these mutants. Since the so^1 and eya^1 mutations eliminate expression of *so* and *eya* transcript and protein in the eye disc, but not elsewhere in the organism, these mutants were defined as eye-specific nulls (Bonini et al., 1993; Cheyette et al., 1994). These findings render them appropriate for the analysis in the eye, especially since so^1 specifically deletes those intronic sequences that contain the *so*10 enhancer. The results in the so^1 and eya^1 mutants clearly show that the maintenance of activity of this element is dependent on the presence of both proteins posterior to the morphogenetic furrow. These data complement the findings obtained in ectopic eyes and fit with the current model previously published on the regulation of retinal differentiation (Bonini et al., 1997; Chen et al., 1997; Czerny et al., 1999; Pignoni et al., 1997). Whereas EY and TOY seem to be required for activation of the *so*10 eye-specific enhancer, an interacting network between SO, EYA, EY and TOY seems to be responsible for maintenance and proper expression in differentiating cells. In ectopic eye development, the genetic pathways governing these events have been shown to involve feedback loops so that all these genes regulate the expression of each other. Our findings indicate that it is true in the normal eye as well.

so is regulated by EY and TOY through the same eye-specific enhancer to specify compound eye development

We have studied in more detail the *so*10 eye-specific enhancer during eye morphogenesis. These studies demonstrate that this element, which is deleted in the so^1 mutant is regulated by both Pax6 proteins in *Drosophila*. Using gel shift and DNase footprinting experiments we demonstrated that TOY and EY are able to bind specifically to this enhancer in vitro. Furthermore, we showed that *so*10 is also activated ectopically by *toy* in an ey^2 mutant background suggesting a direct activation by *toy*. Our rescue assays performed with the different *so*10 mutant drivers, which are exclusively expressed in the compound eye field but not in the ocelli, reflect the requirement of the EY/TOY binding sites and to a lesser extend those of TOY for compound eye development. The phenotype obtained with the *so*10$^{EY/TOYmt}$ driver nicely parallels the variability in eye size observed in *ey* mutants (Halder et al., 1998). Moreover, expressing *toy* in the *ey* domain of the eye disc by using the *ey* enhancer Gal4 driver, established that *toy* could partly complement the lack of *ey* activity. These findings lead us to propose that the variable eye phenotype seen in ey^2 mutant is due to the presence of *toy* rather than a residual expression of *ey*. In this respect, we did not succeed in detecting any *ey* transcript or protein in the ey^2 mutant eye disc (Callaerts et al., 2001; Punzo et al., 2001; Quiring et al., 1994). Furthermore, the *ey* null mutant $ey^{J5.71}$ also has a variable eye phenotype (Callaerts et al., 2001). Thus, the two Pax6 proteins share similar biological properties to direct eye development.

The TOY binding sites are necessary and cooperate with other *cis* regulatory sequences to ensure ocelli development

The incomplete rescue obtained with the *so*10 driver led us to search for additional *cis* regulatory sequences. We found that the *so*9 fragment adjacent to the *so*10 enhancer, which has been shown not to be inducible by EY (Niimi et al., 1999), contains *cis* regulatory sequences acting in combination with the *so*10 eye-specific enhancer to ensure correct expression in the eye. This combined fragment (*so*7=*so*10 + *so*9), which encompasses the so^1 deletion, is able to ensure complete rescue of the compound eye and partial rescue of the ocellus when used as a Gal4 driver. The mutated *so*7 drivers confirmed our previous findings on the EY/TOY binding sites of the *so*10 enhancer with regard to compound eye formation and further indicated a functional difference for the TOY binding sites since the latter are absolutely required for ocellar development. This further demonstrates that TOY requires additional transcription factors present in the eye disc for its proper function. Thus, the different biological properties of EY and TOY previously described are not limited to their DNA binding specificities (Czerny et al., 1999) but extend to their ability to cross talk with different sets of proteins.

Beside its role in ocelli specification, the *so*9 fragment also exerts a finely tuned effect on the *so*10 activity in the compound eye, since *so*7$^{EY/TOYmt}$ is more down-regulated in the eye field than *so*10 (Fig. 6C). These expression patterns are in line with the rescue experiments; there is no rescue of the compound eye with *so*7$^{EY/TOYmt}$ (Fig. 6G), whereas *so*10$^{EY/TOYmt}$ gives at least a partial rescue (Fig. 5D). The compound eye is fully rescued with the wild-type *so*7 driver and partly rescued with the *so*10 driver.

Common sites for distinct biological functions

Using our in vitro approach, we could not define any EY-specific sites since the sites 1, 2 and 5 are equally bound by EY and TOY. These sites might be more EY-specific since the residual expression of the *so*10^{TOYmt} is lost in ey^2 eye discs. In

addition, *so*10 expression in *ey* mutant eye discs is similar to the *so*10$^{EY/TOYmt}$ expression in wild-type eye discs. Nevertheless, we cannot exclude the possibility that both proteins bind in a complex combinatorial arrangement to these sites in a wild-type eye context. Alternatively, it is also possible that these sites will be occupied by either EY or TOY depending on which protein is present in the nucleus. In this respect, the efficiency of the ocellar rescue is relevant. The rescue obtained with the *so*7$^{EY/TOYmt}$ lacking binding sites 1, 2 and 5 is reduced qualitatively (ocelli smaller) and quantitatively (40% of the flies show a rescue) as compared to the rescue efficiency obtained with the wild-type enhancer (80% of rescue efficiency). This strongly suggests a requirement of all five binding sites for TOY to ensure full enhancer activity in the ocelli.

The footprinting experiment did not reveal any binding of the EY-PD onto the binding sites 3 and 4. Since Czerny et al. (Czerny et al., 1999) demonstrated a reduced binding activity for the EY-PD when compared to the TOY-PD on the TOY binding sites located in the *ey* enhancer, we cannot exclude that the EY-PD might also bind weakly to the TOY-BS 3 and 4 of the *so* enhancer in vivo.

Two *Pax6* genes with distinct biological functions

All animals, ranging from flatworms to mammals, analyzed so far, have a *Pax6* gene which from our current state of knowledge, is universally required for eye specification. In contrast to vertebrates, where generally a single *Pax6* gene gives rise to several differentially spliced transcripts, *Drosophila* and other holometabolous insects have two *Pax6* genes, raising the question of functional redundancy. Gene duplication and subsequent divergence of developmental control genes is a major driving force in evolution increasing the diversity and complexity of the organisms. A second mechanism for recruiting additional genes into a developmental pathway is enhancer fusion (Gehring and Ikeo, 1999). The acquisition of new *cis*-regulatory elements represent an important mechanism for functional diversification (Bouchard et al., 2000; Greer et al., 2000; Hanks et al., 1995; Suda et al., 1999; Xue and Noll, 1996). Our findings strongly support both of these hypotheses since *toy* is able to rescue the eye development in an *ey* mutant when expressed in the *ey* domain. The finding that *ey* and *toy* exhibit different expression patterns during embryogenesis might account in part for their functional biological diversity (Czerny et al., 1999; Kammermeier et al., 2001). In the eye, both genes are co-expressed, except for the ocellar territory where only *toy* is expressed (Czerny et al., 1999). In addition, it has been proposed that TOY and EY diverged to regulate different sets of target genes because of a N14G mutation that changes the DNA binding specificity of the PD domain of *ey* (Czerny et al., 1999). Indeed, using the *so*10 regulatory element we found that TOY does not bind to the same sequences as EY, but interestingly, TOY and EY regulate the same target enhancer in different cells. The phenotypes obtained in our rescue experiments using either the EY/TOY or TOY binding site mutated enhancers, nicely parallel the phenotypes observed in those mutants. The *ey* null mutant still has ocelli but lacks compound eyes. Interestingly escapers from the recently isolated *toy* mutant in our laboratory (*toy*$^{G7.39}$) exhibit no eye reduction whereas the ocelli are partially missing (S. F., U. Kloter and W. J. G., unpublished). Therefore, removal of the common target gene of both Pax6 proteins in the eye (e.g. *so*^{1} mutant) consequently leads to a loss of both compound eyes and ocelli. Therefore, we propose that one of the developmental programs of *toy* is in part to specify ocellar development in addition to head formation, since *toy* mutants generated are characterized by pupal lethality, pharate adults lacking half of the head or the entire head capsule (Kammermeier et al., 2001). Thus, we can propose that the *so* gene is regulated by *toy* to specify the ocelli and by *ey* to specify the compound eyes during larval development.

Our analysis of *ey* and *toy* allows us to dissect the evolutionary changes after the gene duplication event that has happened during insect evolution. First, the *cis*-regulatory regions of the two genes have diverged, leading to both temporal and spatial changes of expression; *toy* is expressed much earlier than *ey* during embryogenesis, whereas *ey* is not expressed in the ocellar region of the larval eye disc (Czerny et al., 1999). Second, the protein coding regions of the two genes have diverged, most importantly in the paired domain where asparagine 14 which is present in most *Pax6* homologs, has been mutated in *ey* to glycine, which changes the DNA binding properties of the protein significantly (Czerny et al., 1999). Third, the positive autocatalytic feedback loop found in vertebrates for their single *Pax6* gene (Chow et al., 1999; Grindley et al., 1995; Okladnova et al., 1998; Plaza et al., 1995), has evolved into a heterocatalytic control loop in which *toy* transcriptionally activates *ey* by binding to the eye-specific enhancer of *ey* (Czerny et al., 1999; Hauck et al., 1999). Fourth, both *toy* and *ey* cooperate in differentially regulating the *so* target gene, reflecting the fact that earlier in evolution *so* was regulated by a single *Pax6* gene. These findings strongly support the hypothesis of intercalary evolution (Gehring and Ikeo, 1999) showing that the *ey* gene has been intercalated into the eye developmental pathway between *toy* and *so*. The observation that *toy* activates *ey* in the eye progenitor cells of

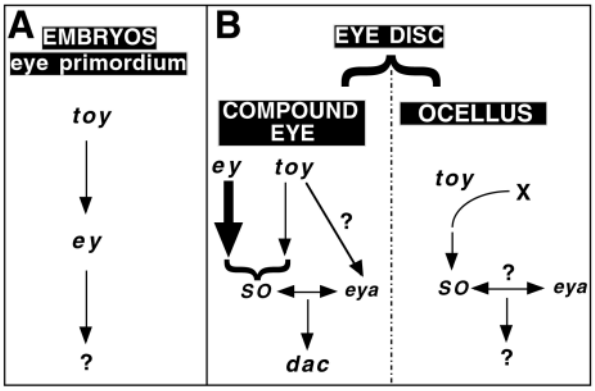

Fig. 7. Eye specifying gene hierarchy depends on the cell type and the stage of development. The network of regulatory genes specifying eye development is modified based on this study and other recently published results. (A) In the embryonic eye precursor cells, *toy* directly activates *ey* through the *ey*-enhancer (Czerny et al., 1999) without *ey* activating its downstream genes *so/eya* (Kumar and Moses, 2001). (B) In the eye disc of late L2 and early L3 *ey* and *toy* directly regulate *so* in a complex network by using different sites on the same enhancer element in different sets of cells to direct the development of the compound eyes and the ocelli.

the embryo, where neither *so* and *eya* are expressed (Kumar and Moses, 2001), indicate that *toy* and *ey* are acting high up in the genetic hierarchy leading to eye development.

Our current model of eye development (Fig. 7) shows that the eye specifying genes are expressed and sequentially regulated during different stages of development. Our study demonstrate a distinct role for TOY and EY in *so* regulation to specify ocelli and compound eye development, respectively.

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